# Project 3
# Cardiovascular Toxicity of Concentrated Ambient Fine, Ultrafine and Coarse Particles in Controlled Human Exposures
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ABSTRACT

Using our controlled particle exposure facility, we have demonstrated that short-term exposures to fine Concentrated Ambient Particles (CAPs) + ozone ($O_3$) cause acute conduit artery vasoconstriction and are associated with increased diastolic blood pressure in healthy adults. Both of these findings are associated with the organic carbon component of the particulate matter (PM). This proposal aims to further examine the size fractions, components, and sources of PM responsible for these cardiovascular physiologic responses. A new state-of-the-art ambient PM exposure facility (to be built at the University of Toronto in collaboration with Harvard School of Public Health) will allow us to examine responses to fine, ultrafine and coarse CAPs, in downtown Toronto, Canada. To gain insight into these responses, cardiovascular outcomes in the proposed study will include not only our more established physiologic outcomes (brachial artery diameter and blood pressure), but also complementary biological measurements including cardiovascular hemodynamics, autonomic function (e.g., HRV), markers of systemic inflammation (e.g., CBCs, IL-6, CRP) and endothelial dysfunction (endothelins).

We propose to expose 50 healthy adults to fine, ultrafine and coarse CAPs, and particle-free (filtered) air. Each participant will receive 4 exposures in random order, separated by at least two weeks. Cardiovascular outcomes will be measured both pre-, post- and 24 hrs post-exposure and will include measures of: brachial artery diameter; flow- and nitroglycerin-mediated dilatation by ultrasonography; stroke volume (SV) and cardiac output (CO) by echocardiography; blood pressure (BP), and; venous blood CBCs, IL-6, CRP and endothelins. Also, during exposures, continuous measurements will be performed using 24-hr Holter monitors of beat-to-beat arterial BP using a Finometer monitor, including calculated determinations of SV, CO and systemic vascular resistance and HRV. Each PM size fraction exposure will be characterized for particle mass, number, diameter, size and composition (inorganic ions, trace metals, organic and elemental carbon and black carbon). Gaseous co-pollutants (carbon monoxide, $CO_2$, NO, $NO_2$, $SO_2$, $O_3$), temperature and humidity will be monitored continuously during the exposure experiments. In addition, on the days before and after exposures, 24-hr measurements will be conducted for each participant using a multi-pollutant personal sampler. **REVISION – No longer being done.**

For each of the observed biological effects, repeated measures ANOVA models will be employed to assess differences among exposures. These models will contain a random effect for subject and a categorical variable for the four exposure treatments (fine, ultrafine, coarse, CAPs and particle-free air). To assess exposure-response relationships between biological outcomes and CAPs mass or individual component concentrations,

single pollutant analyses will be conducted in which a separate linear mixed regression model will be used for each exposure parameter. These models will use biologic response as the dependent variable, subject as a random effect, and either particle mass, number, diameter or component as the exposure metric in the model allowing for a separate slope for each size fraction. Hierarchical linear models will be developed to account for the multiple levels of data, including measurements taken at different time points within an exposure, for a subject. We expect to find physiologic responses consistent with vascular narrowing (increased BP, decreased brachial artery diameter) in response to all three CAPs size fractions, as compared to particle-free air. Also, we expect that the cardiovascular responses may vary among fine, ultrafine, and coarse size fractions.

## 1. OBJECTIVES

The proposed project will examine the acute cardiovascular effects of fine, ultrafine and coarse CAPs in healthy adults, using a controlled particle exposure facility, in Toronto. Fifty subjects will each receive three CAPs exposures (3 size fractions) and a particle-free air exposure, in random order. Cardiac vascular/physiologic outcomes will be measured both pre-, post- and 24 hrs post-exposure, and will include measures of: i) vascular dysfunction (brachial artery diameter, flow- and nitroglycerin-mediated dilatation by ultrasonography; stroke volume and cardiac output by Doppler, 2-D echocardiography); ii) blood pressure and; iii) markers of systemic inflammation (CBCs, IL-6, CRP, endothelins) in blood taken pre-, post- and 24 hrs post-exposure. We will also perform continuous (in-exposure) measurements of: beat-to-beat arterial BP using a Finometer monitor, including calculated determinations of cardiac output, stroke volume and systemic vascular resistance (SVR) and; measures of cardiac autonomic dysfunction (HRV using 24 hr Holter monitoring). The specific hypotheses to be addressed by this proposed research are the following:

- Acute human exposures to CAPs of ultrafine, fine and coarse size fractions result in cardiovascular responses consistent with vascular narrowing, vascular/autonomic dysfunction, inflammation, and/or endothelial activation compared to filtered air (control) exposures.

- Associations between CAPs and cardiovascular responses differ by particle size fraction and composition.

## 2. INTRODUCTION

2.1. Air Pollutants and Morbidity and Mortality from Cardiovascular Diseases: Studies have consistently demonstrated an association between ambient particulate matter (PM) and increased short- and long-term morbidity and mortality from cardiopulmonary diseases[1,2], especially for particles smaller than 2.5 μm in diameter, $PM_{2.5}$[3,4]. Furthermore, PM levels have been associated with morbidity and mortality from specific cardiovascular diseases such as myocardial infarction[5], congestive heart failure and coronary artery disease[6,7]. Coronary artery disease is now considered, in large part, an inflammatory process. Indeed, current thinking suggests that the cardiovascular effects of PM inhalation can be mostly attributed to systemic or vascular inflammation, oxidative

stress, endothelial activation, or autonomic dysfunction. There is growing evidence implicating each of these potentially related processes.

2.2. Inflammation, Oxidative Stress, and Endothelial Activation: PM exposures have been associated with elevations in blood viscosity, fibrinogen, CRP, and red blood cells[5,8-10]. Animal studies have identified similar systemic effects of PM such as systemic bone marrow responses[11], the progression of atherosclerosis,[12] and an increase in plasma endothelins[13] and oxidative stress metabolites[14].

2.3. Fine, Ultrafine and Coarse CAPs: The main objective of this study is to investigate responses of established cardiovascular endpoints in human studies with fine, ultrafine, and coarse particle exposures. The ultrafine fraction (<0.1 μm) is thought to be toxic because it largely consists of combustion by-products and heavy metals[15,16]. There is also evidence that ultrafine particles, due to their size, can enter the circulation directly from the lungs and

3-1

potentially exert systemic effects including tendency to thrombosis[17,18]. Some studies have suggested that ultrafine particles are more toxic than larger particles of the same composition[19], due to their lung deposition efficiencies[20]. Furthermore, ST-segment depression was associated with ultrafine exposures in a Finnish repeated-measures study of adults with coronary artery disease[21]. Despite the potentially high toxicity of ultrafine particles and their inherent ability to pass directly into the vasculature, evidence for cardiovascular effects of acute ultrafine particle exposures is limited compared to that for fine particles. Studies are needed that directly assess ambient ultrafine particles in relation to health outcomes. The development of the ultrafine particle concentrator (Particle Technology and Monitoring Core, Section 3.1.2) is an important advance that will facilitate these studies.

Fine particles account for most of the atmospheric PM mass and are the least scavenged by atmospheric processes[22]. Therefore, they have the longest atmospheric residence time and thus the greatest potential for human exposure under typical ambient conditions. Some epidemiological studies have specifically identified $PM_{2.5}$ (albeit usually containing some amount of ultrafine particles) as the fraction most strongly associated with adverse health effects[3]. Extensive testing in animals and humans has also revealed a number of adverse cardiovascular responses, including ECG and HRV abnormalities in dogs[23], arterial vasoconstriction[24] and increased blood pressure[25] in humans, and the progression of atherosclerosis in rabbits[12]. Results of controlled exposures in our lab suggests an association between particulate exposure and increased blood pressure[25] in humans. Toxicological studies comparing the effects of fine particle exposures to those of ultrafine and coarse particles are of paramount importance in our efforts to differentiate the size-specific cardiovascular effects of PM. Coarse particles are also a heterogeneous mixture of materials that includes re-suspended road dust, bioaerosols and trace elements[26]. In

vitro and animal studies have suggested that coarse-mode biological materials, such as endotoxin and glucans, may be related to specific health effects[27-30]. There is also a significant amount of epidemiological evidence associating $PM_{10}$ mass, which contains coarse particles, with adverse cardiopulmonary outcomes[31-33]. The recent development of a coarse particle concentrator is a critical technological advance allowing for the investigation of coarse particle effect (Particle Technology and Monitoring Core, Section 3.1.2). To date, a study of human exposure to concentrated ambient coarse particles has already been conducted[34], demonstrating mild decreases in heart rate variability in the absence of significant decrements in pulmonary function or inflammation. Further studies on this size fraction are clearly needed. The proposed study will be the first to compare responses in the same person exposed to all three size fractions of real-world PM.

3. PRELIMINARY RESULTS

3.1. Autonomic Dysfunction: $PM_{2.5}$ has been shown to decrease overall heart rate variability in humans[38-40], which suggests a reduction in vagal tone and/or an increase in sympathetic drive. Black carbon (BC), a marker of traffic particles, was more strongly associated with reduced HRV than $PM_{2.5}$ in a panel study of 27 elderly subjects (aged 61-89) living in an apartment building in Boston[41]. Each subject was seen weekly for 12 weeks during the summer of 1999. BC was more strongly associated with reduced heart rate variability than non–traffic particles (Table 1). Carbon monoxide showed similar patterns of association as BC, which disappeared after controlling for black carbon. Carbon monoxide was not a confounder of this association. 3-2

| Variable | SDNN | r-MSSD |
|---|---|---|
| BC° | -4.6 (-2.0, -7.2) | -6.1 (0, -11.9) |
| BC* | -5.1 (-1.5, -8.6) | -10.1 (-2.4, -17.2) |
| $PM_{2.5}$° | -3.4 (0.6, -7.3) | -7.4 (1.6, -15.5) |
| $PM_{2.5}$* | -2.6 (0.8, -6.0) | -10.1 (-2.8, -16.9) |
| Secondary PM° | -0.5 (2.8, -3.7) | -3.0 (4.8,-10.3) |
| Secondary PM* | -0.9 (2.5, -4.1) | -6.4 (0.6, -12.9) |

° 1 hour average *24–hour average
All models control for subject, temperature, day of week, hour of day, medication use that day, and time trend. Secondary PM = effects due to everything other than BC. SDNN = standard deviation of RR intervals;
r-MSSD= root mean square of successive differences in RR intervals
Table 1: Percent Change in Heart Rate Variability Associated With an Interquartile Range Increase in Particle Exposure

3.2. Arterial Vasoconstriction and Increased Diastolic Blood Pressure: In an effort to understand the physiological changes underlying the association between increased acute cardiovascular events and air pollution exposure, we investigated the effect of fine CAPs

+ added $O_3$ on human vascular function[24]. We were interested in sudden arterial vasoconstriction because it is capable of triggering a variety of acute cardiovascular events, such as cardiac ischemia and myocardial infarction[42] – outcomes seen in toxicological and epidemiological studies. The function of the arterial endothelium was also examined, because acute endothelial dysfunction plays a pivotal role in the development of atherosclerosis[43] and is now established to independently predict an adverse cardiovascular prognosis[44,45]. We demonstrated that a 2-hr exposure of healthy adults to fine CAPs (150 $\mu g/m^3$) + $O_3$ (120 ppb), both environmentally relevant concentrations[46], caused a significant brachial artery vasoconstriction (reduction in brachial artery diameter (BAD), Table 2).

Table 2: Vascular Responses (mean ± SD) Following Particle-Free (Filtered) Air and CAP + $O_3$ Exposures[24]

| | Filtered Air | | | CAP + $O_3$ | | | |
|---|---|---|---|---|---|---|---|
| TEST | Pre | Post | Δ (post–pre) | Pre | Post | Δ (post–pre) | p* |
| BAD (mm) | 3.89 ± 0.68 | 3.90 ± 0.68 | 0.01 ± 0.18 | 3.92 ± 0.65 | 3.82 ± 0.62[†] | -0.09 ± 0.15 | 0.03 |
| FMD (%) | 3.61 ± 4.27 | 3.57 ± 6.49 | -0.03 ± 6.63 | 4.22 ± 4.52 | 4.52 ± 3.69 | 0.29 ± 4.11 | 0.88 |
| NMD (%) | 14.69 ± 6.40 | 18.15 ± 8.94 | 3.46 ± 7.92 | 14.88 ± 5.25 | 18.76 ± 6.12 | 3.87 ± 5.43 | 0.83 |

* p values for (filtered air Δ) versus (CAP + $O_3$ Δ), two-tailed, paired t-test

[†] p = 0.007; for post-BAD (CAP + $O_3$) vs. pre-BAD (CAP + $O_3$), two-tailed, paired t-test
FMD = flow-mediated dilatation; NMD = nitroglycerin-mediated dilatation
3-3
We were able to demonstrate that exposures to fine CAPs with a higher organic carbon content were associated with greater brachial artery constriction[52] as compared to fine CAPs exposures with less organic carbon content.

We have also demonstrated that fine CAPs exposure results in pulmonary arterial vasoconstriction in animals[47]. Specifically, male Sprague-Dawley rats were exposed to CAPs or particle-free air for 5 hrs/day for 3 consecutive days. In this study the lumen/wall ratio (L/W) was morphometrically determined on transverse sections of small pulmonary arteries. A decrease in L/W ratios was observed, which was associated with an increase in $PM_{2.5}$ mass, silicon, lead, sulfate and elemental and organic carbon concentrations. More recently, we reported that exposure to 150 $\mu g/m^3$ fine CAPs + 120 ppb $O_3$ was associated with a statistically significant elevation of 4.2 mm Hg in diastolic

blood pressure in 35 non-smoking healthy adults during the course of exposure[25]. The biological interpretation of this finding constitutes the subject of a paper in preparation.

As with our brachial artery diameter findings, we also demonstrated that exposures to fine CAPs with a higher organic carbon content was associated with a greater increase in diastolic blood pressure[71] as compared to fine CAPs exposures with less organic carbon content. Systolic blood pressure and heart rate were unaffected. Our findings also highlighted the importance of the conditions under which blood pressure measurements are taken. The elevation in diastolic blood pressure was observed only when subjects were inside the chamber, where factors that affect blood pressure such as activity level and temperature were tightly controlled. Measurements of blood pressure taken after the subjects had exited the chamber (and therefore were no longer at rest) were not different between pollutant and filtered air exposures.

Three published epidemiological studies support this finding[48-50], including our own work demonstrating increases in diastolic blood pressure related to the mean of the previous 2 to 5-day $PM_{2.5}$ concentrations in 66 cardiac rehabilitation patients in Boston (Table 3)[50,51]. The lack of personal exposure measurements and the fact that patients were in a clean, particle-free air environment during testing limited the potential to look at more immediate blood pressure effects of local ambient pollution.

Finally, we have recently begun to examine the relative effects of fine CAPs versus ozone, as well as potential mechanisms of the brachial artery and blood pressure responses. The former objective is being carried out at the Human Exposure Facility (Toronto), where 50 healthy adults receive 4 exposures (7 subjects already completed): filtered air, 150 $\mu g/m^3$ fine CAPs, 120 ppb $O_3$, and fine CAPs + $O_3$. Potential mechanisms are being tested at the University of Michigan,[3-4] where 50 healthy adults are receiving fine CAPs + $O_3$ exposures after being pretreated with placebo, Bosentan (an endothelin receptor blocker) and vitamin C (an antioxidant).

3.3. Cardiovascular Function and Hemodynamics: We now wish to gain insight into the potential biological mechanisms responsible for the observed brachial artery constriction in our previous human exposure experiments[24]. Echocardiography will be used to measure cardiac output and stroke volume changes in response to different size fractions of ambient PM. Using Doppler and two dimensional echocardiographic techniques[35], stroke volume and cardiac output can be measured easily, noninvasively, and reliably[36]. This technique is especially well-suited for the assessment of serial changes in these hemodynamic parameters in response to intervention and has been used to assess the hemodynamic effects of drug therapy, changes in cardiac output during pregnancy and the puerperium, and changes in cardiac output with various modes and programming of pacemakers. Conceptually, blood flow (Q) through the heart is similar to flow through a tube of known diameter and can be estimated by the equation: Q = (cross sectional area of the tube) x (velocity).

Although, theoretically, any of the cardiac valves can be used for the calculation, the aortic valve has been demonstrated to be the most accurate and reproducible for several reasons [37]. First, the cross-sectional area of the aortic valve is circular and the calculation more straightforward (unlike the mitral and tricuspid valves which are elliptical). Second, the diameter (D) of aortic annulus is easily measured in the parasternal long axis view and the area calculated as $Area = \pi \, (D/2)^2$. Finally, due to the fibrous nature of the aortic annulus, the diameter does not change significantly during the cardiac cycle.

We will be able to determine whether the measured change in sympathetic nervous system (SNS) tone is associated with alterations in BP due to increasing cardiac inotropy and/or cardiac output. We will also be able to relate any changes in cardiac output/stroke volume to changes in BP, in order to determine whether they play a role in expected (and previously observed) increases in BP. The continuous measurement of finger blood pressure (Finometer) also allows for non-invasive determination of systemic vascular resistance (SVR) – a desirable endpoint to be studied following the three CAPs exposures compared to the particle-free air exposure.

These outcome measures can then be related to the changes in HRV/SNS tone and the observed reduction in BAD/FMD by vascular ultrasound. The relation of changes in SVR to BP can provide insight into whether a primary vasoconstrictive mechanism is responsible for the increased diastolic BP. Furthermore, the correlation between changes in HRV/SNS tone and vascular function (reduction in BAD and/or FMD) with the change in SVR will provide insight into whether any observed increase in SVR is primarily due to increased SNS tone and/or direct vascular dysfunction. The simultaneous measurement of both vascular function and autonomic tone along with determining continuous BP, cardiac output and systemic hemodynamics will provide an unprecedented ability to investigate the biological cardiovascular responses to different PM size fractions, and will allow for the determination of their relative (vascular versus autonomic changes) significance in altering cardiovascular physiology. [3-5]

## 4. APPROACH      (PROTOCOL – REVISED AS OF MARCH 2006)

4.1. Study Design: 50 healthy adults (25 males and 25 females), will be exposed to fine, ultrafine and coarse CAPs and particle-free (filtered) air. Each subject will receive 4 two-hour exposures in random order and that are separated by at least two weeks. Each exposure will take place in the morning, commencing at around 10 a.m. Cardiovascular outcomes will be measured both pre-, post- and 24 hrs post-exposure, as detailed in sections 4.3 and 4.4.

This is a randomized block design study that is double-blinded and balanced across the 4! = 24 possible orderings of the exposure sequences for both male and female subjects. The four exposures will include: i) particle-free air (HEPA filtered); ii) fine CAPs (150 $\mu g/m^3$); iii) ultrafine CAPs ($\Box \, 10^5$ counts/cm$^3$ – a concentration factor of ~ 50 times the Toronto ambient counts) and; iv) coarse CAPs (150 $\mu g/m^3$). The exposure conditions for which we observed arterial vasoconstriction and an increase in diastolic blood pressure included both 150 $\mu g/m^3$ ambient fine particles with 120 ppb added ozone, a combination that commonly occurs in the ambient air. Preliminary results of our ongoing study, which

is examining the independent and combined effects of CAPs and $O_3$, will be used to inform subsequent exposures. If, for example, the addition of $O_3$ is found to be necessary for eliciting responses, it will be added to the treatment exposures.**REVISION – No longer being added.**

4.2. Controlled Exposures: This study will utilize the Harvard Ambient Particle Concentrators to generate controlled human exposures to fine, ultrafine and coarse particles[53-55]. The exposure facility, which will be completed by the spring of 2005, is being constructed through an infrastructure grant (from the Canadian Foundation for Innovation to the Southern Ontario Centre for Atmospheric Aerosol Research) at the University of Toronto, in collaboration with the Harvard School of Public Health (HSPH). The fine, coarse and ultrafine concentrator technologies are presented in the Particle Technology and Monitoring Core, section 3.1.2. Particles will be drawn from an inlet located 10 m above a busy street in downtown Toronto (a major urban center with a population of over 2.5 million people). Thus, traffic particles are a major contributor to the ambient PM levels at this site. The CAPs air stream will be delivered directly to the subject who will be seated (inside a modified plethysmograph enclosure) at rest and breathing freely (no mouthpiece) via an "oxygen type" facemask covering his/her nose and mouth[56]. Gaseous co-pollutant levels from the ambient inlet air, including NO, $NO_2$, $O_3$, carbon monoxide, $CO_2$ and $SO_2$, will be monitored continuously during CAPs exposures. In previous studies[24,25,56] the ambient gaseous concentrations have not significantly differed between exposures (CAPs versus filtered air) and will not likely contribute to any differences in response.

4.2.1. Exposure Characterization: Air will be drawn from the CAPs air stream for particle and gas measurements. Continuous particle and gaseous co-pollutant measurements will be performed for every CAPs and particle-free (HEPA filtered) exposure. Integrated particle samples will be collected only for CAPs exposures. Measurement analyses will include: particle mass, size, number, diameter concentration and composition (elements, EC/OC, BC, $SO_4^{2-}$, $NO_3^{-}$, $NH_4^{+}$ and organics), $O_3$, $SO_2$, NOx, carbon monoxide, non-methane hydrocarbons (NMHC), $NH_3$, and aldehydes. Section 3.2.4 of the Technology and Monitoring Core presents details on the analytical methods for the above measurements. A co-investigator, Dr. James Scott, will use modified Limulus Amebocyte Lyase (LAL) tests[3-6] for the detection and quantification of bacterial endotoxin and yeast glucans on fine and coarse CAPs exposure filters.

4.2.2. Personal Air Pollutant Monitoring: All subjects will be equipped with personal exposure monitors both the day before and the day after they are exposed. The Harvard multi-pollutant samplers will be used to measure 24 hr $PM_{2.5}$, BC, trace elements and $NO_2$. Personal samples will be sent to HSPH for analysis. Section 3.2.2 of the Particle Technology and Monitoring Core discusses details of the sampling and analysis methods. Personal monitoring will make it possible to control for the effects of the pre- and post-exposures from ambient and micro-environmental sources.**REVISION – No longer being done.**

4.3. Biological Outcome Measurements: A summary of the biological measurements to be collected is provided in Table 4.

Table 4: Summary of primary and secondary endpoint measurements, including method by which they will be obtained

| Effect Assessed | Specific Parameters | Method | Measurement Period |
|---|---|---|---|
| Vascular function/injury | brachial artery diameter*, flow-mediated dilatation, nitroglycerin-mediated dilation | Ultrasound | Pre-, post- & 24 hrs post-exposure |
| Vascular injury | blood pressure* | Oscillometric | Pre-, post- & 24 hrs post-exposure; intraexposure |
| Hemodynamics | blood pressure, systemic vascular resistance, stroke volume, cardiac output | Finometer | During exposure |
| Cardiac Injury | cardiac output, stroke volume | Echo-cardiography | Pre-, post- & 24 hrs post-exposure |
| Pulmonary Function | peak expiratory flow (PEF), tidal volume, respiratory frequency, minute ventilation | Flow turbine | 0-hr and every 30-min during exposure |
| Pulmonary Function | FVC, $FEV_1$, PEF, $FEF_{50}$, $FEF_{75}$ | Pneumotach | Pre-, post- & 24 hrs post-exposure |
| Autonomic function | SDNN, r-MSSD, PNN50, LF, HF, LF/HF | ECG (Holter HRV) | During exposure (also 24-hr monitoring the day before & after) |
| Systemic Effects | complete blood count, IL-6, CRP, endothelin | Venous Blood | Pre-, post- & 24 hrs post-exposure |

* primary outcome (all other secondary outcomes are considered "specific parameters")

4.4. Measurement Techniques

4.4.1. Vascular Function Testing: Brachial artery reactivity testing will be measured in accordance with published guidelines[57], and a previously described methodology[24], using a high resolution Terason™ 2000 ultrasound system (2000 Teratech Corp. http://www.terason.com/)

3-7

with a 7.5 mHz linear array transducer. To minimize extraneous effects on basal brachial artery diameter and reactivity (flow-mediated dilatation, FMD)[58], standardized conditions and protocols will be implemented for each subject prior to and during vascular testing. Subjects will arrive fasting, as postprandial lipemia/glycemia may alter FMD for up to 5 hrs after eating. The time of day for measuring vascular function will remain the same for

each individual (between 10 am and 12 noon), and will be performed in a temperature-controlled room.

A baseline brachial artery diameter (BAD) will be imaged longitudinally by B-mode imaging with the transducer 2 - 10 cm above the antecubital crease on the right arm, and all subsequent images will be obtained at this identical arm location. All images are acquired at the end of each R wave on ECG by a triggered event and stored directly on the Terason computer for later off-line analyses. Peak systolic and end-diastolic blood velocity will be determined in the central brachial artery segment by Doppler imaging at rest (prior to FMD).

Endothelial-dependent flow-mediated dilatation (FMD), defined as the percent change in the brachial artery lumen diameter from baseline in response to reactive hyperemia, will also be imaged. A blood pressure cuff will be inflated to 40 mm Hg above systolic blood pressure, (maximum of 200 mm Hg), over the proximal portion of the ipsilateral upper arm and rapidly deflated after 4 mins of occlusion, creating reactive hyperemia-induced increased blood flow and shear stress in the brachial artery.

Endothelial-independent nitroglycerin-mediated dilatation (NMD), determined as the percent dilatation of the brachial artery 3 mins following 0.4 mg of sublingual nitroglycerin, will be imaged. If systolic pressure is < 100 mm Hg, and/or the subject is lightheaded, orthostatic, or has a history of reaction to nitroglycerin (e.g. hypotension, severe headache), then the NMD procedure will not be performed.

Image analyses will be performed at a later time using the latest commercially available software package by Medical Imaging Applications, Inc (http://www.mia-llc.com). This software uses sophisticated digital edge-detection protocols that significantly reduce the variability in image measurements[59]. The protocol provides the standard 1 min post-release brachial FMD (primary endpoint in this study), as well as further FMD secondary endpoints that may be of relevance (time-to-peak vasodilatation, peak percent vasodilatation, mean 2 min vasodilatation). These secondary FMD measurements provide additional information regarding the functioning of the vascular endothelium and may be superior markers of endothelial function to traditional 1 min post reactive hyperemia FMD[57].

In a separate study, we analyzed the test-retest reproducibility of FMD in 25 healthy adults who had their vascular function (FMD) measured on two separate occasions at least one week apart (intra-individual reproducibility). The mean population standard deviation of the FMD measurements was 3.5% (with a mean FMD of 7.0%). The test-retest correlation coefficient was r=0.51, p=0.03. The mean coefficient of variation for FMD was 50%. These findings are similar to other reports in the literature[58,60] and to the published guidelines for brachial FMD measurement[57], and demonstrate that the variability in FMD determination is NOT due to the measurement technique, ultrasound resolution/capabilities (theoretical axial resolution of approximately 0.08-0.1 mm), and/or analysis software, but rather it is a direct consequence of the[3-8] normal day-to-day alterations in basal arterial tone/function (due to physiology and/or environmental factors).

4.4.2. Echocardiographic Measurements of Stroke Volume and Cardiac Output: Using Doppler and two dimensional echocardiographic techniques, stroke volume and cardiac

output will be measured. In practice, cardiac flow (stroke volume) is computed as the product of the cross sectional area of a valve calculated from measurements of valve diameter on the 2-D echocardiogram and the instantaneous velocity time integral of that valve determined by Doppler. The aortic velocity waveform is assessed using a pulsed-wave Doppler with the sample volume placed at the aortic valve annulus in the apical three or five chamber view and the velocity time integral is traced either on-line or later off-line using commercial software. Instantaneous cardiac output is calculated as follows:

Cardiac Output (CO) = Stroke Volume x Heart Rate

CO determined by echocardiographic methods has been demonstrated to have good correlation (r = .75 to .98) with thermodilution CO and with CO determined by the Fick method. Inter-observer variability of the measurement of aortic flow using this method ranged from 3.5 ± 2.2% to 5.4 ± 3.4% and intra-observer variability ranged from 1.9 ± 1.8% to 3.2 ± 2.9% in a study of normal patients[37,61].

4.4.3. Continuous Blood Pressure Monitoring: Continuous beat-to-beat blood pressure will be determined non-invasively by the Finometer (Finapress Medical Systems, www.finapres.com) arterial blood pressure and hemodynamic monitor[62]. This device provides a measurement of intra-arterial finger blood pressure based upon the validated volume-clamp method of Penaz[62-65]. The Finometer is also capable of providing accurate brachial beat-to-beat blood pressure measurements by compensating for the arterial pressure waveform distortion of the finger by measuring blood pressure in the arm by oscillometric technique and by correcting for hydrostatic pressure differences between the finger and arm. The Finometer will also provide off-line software calculations of stroke volume (SV), cardiac output (CO), and systemic vascular resistance (SVR) by the Modelflow method based upon the finger arterial pressure and waveform analyses[63,65]. This method digitally computes an aortic flow waveform based upon the digital arterial waveform and a Windkessel model of the aortic input impedance, arterial compliance, and SVR. The accuracy of the system has been validated by both the Association for the Advancement of Medical Instrumentation and the British Hypertension Society protocols[64]. The device has recently demonstrated an excellent capability and sensitivity to accurately determine small, acute changes in cardiovascular hemodynamics such as blood pressure, SV, CO, and SVR[63]. The Finometer is a small, portable non-invasive device that will be attached to each subject just prior to entering the air pollution exposure chamber. The device will be turned on when the patient enters the chamber and will continuously monitor finger arterial waveforms, beat-to-beat blood pressure, and systemic hemodynamics throughout the 2-hour long exposure period.

Automated oscillometric cuff blood pressure measurements will also be recorded before and after, as well as every 30 mins during exposures. At each time point (0 min, 30 min, 60 min, 90 min, 120 min) three blood pressure measurements separated by 15 seconds will be taken and recorded by the Exposure Technologist. The first measurement will be discarded (i.e., "white coat" effect) and the average of the other two will be used in the statistical                                                                                                    analysis.

3-9

4.4.4. Blood Collection: Venous blood will be collected in EDTA tubes from all study participants prior to, and 3 and 24-hrs after each exposure. Whole blood will be used to

make smears for cellular differentiation and complete blood cell counts. Other samples will be processed and stored for the analysis of markers of oxidative stress and inflammation (IL-6, C-reactive protein, endothelins). Stored samples will be sent to HSPH and analyzed in accordance with Projects 1 (Section 4.3.3) and 2 (Section 4.3.2).

4.4.5. Pulmonary Function: The methodology for spirometry (flow-volume curve) has been described previously[66] and follows established standardized procedures[67]. This will be used to determine whether exposure to different size fractions of CAPs is associated with decrements in lung function. However, in our previous exposure studies, no CAPs-related changes have occurred[56]. For safety reasons, subjects will also be monitored for changes in peak expiratory flow using a peak flow meter during exposure, after one hour of exposure and if any breathing discomfort occurs (see section 4.9).

4.4.6. Heart Rate Variability: Continuous heart rate monitoring will be performed using a 5-lead Holter monitor from Cardio Data Systems. Each subject's skin will be prepared and electrodes will be placed in a modified $V_1$ and $V_5$ position. Holter monitoring will take place for the 24-hr periods before and after the exposure, to coincide with the personal exposure monitoring time-frame.**REVISION – no longer being done.** Immediately prior to exposure, after the exposure and 24 hrs later, we will collect 10-minute resting HRV readings. Holter monitoring will also take place over the course of exposure for the purpose of comparing during-exposure HRV changes with alterations in cardiovascular hemodynamics (also measured concurrently and continuously). Holter data will be transferred to CD/DVD and shipped to the laboratory of Dr. Peter Stone at HSPH for analysis.

4.5. Data Handling

4.5.1. Vascular Function, Echocardiography and Hemodynamic Measurement Analysis: The results of each subjects' vascular testing images (FMD, BAD, NMD), and cardiac ultrasound images/doppler results will be stored on the Terason laptop hard drive and be backed up on CDs. Hemodynamic data will be collected continuously (and stored), then directly downloaded to a personal computer. Modelflow software (Beatscope 1.1 program, Finapress Medical Systems) will be used to determine all hemodynamics (continuous blood pressure, CO, SVR, SV) that occurred throughout the 2 hr exposure period based upon the subjects' gender, age, body mass, and height. We will also collect 10 min resting data immediately prior to, after and 24 hrs post-exposure. The CD images and hemodynamic data will be sent to the University of Michigan Vascular Core Laboratory for analysis (http://www.med.umich.edu/endothelial /index.htm) by Drs. Robert Brook and Julie Kovach.

4.5.2. Heart Rate Variability: Data from the CD/DVD will be shipped to the Harvard Ambulatory Electrocardiographic (AECG) Laboratory, and transferred to the hard drive of the Del Mar/Reynolds Pathfinder Holter computer for analysis. Each recording will be edited for artifact and beat classification and then analyzed for heart rate variability (time domain),

3-10

arrhythmia counts, ST-segment baseline values, and ST-segment deviation, using standard, validated algorithms. Frequency domain analyses may also be performed. Data will be analyzed using 5 minute epochs and using the entire recording as one epoch. A

hard copy report of all Holter data will be generated and kept in a file folder and stored in a dry safe, locked storage room. The Holter endpoint data will be stored electronically and will be transferred electronically to the HSPH for data checks and management, prior to inclusion in the study dataset in Toronto. The analyzed data will be archived in electronic format as well, using three CD/DVDs (1 for storage at HSPH, 1 offsite and 1 to remain in the AECG Laboratory). The Holter technician will use Reynolds software tools to process the ECG data under the training and supervision of Dr. Stone, with input from Dr. Gold. Exclusion criteria for HRV analysis will include unstable angina, atrial flutter, atrial fibrillation, and paced rhythm. Regions of noise and artifact will be eliminated. After correction, only normal-to-normal (NN) intervals between 150 and 5000 ms with NN ratios between 0.8 and 1.2 will be included for analysis of heart rate variability. The following time domain heart rate variability outcomes will be measured for the entire duration of the protocol: (1) the standard deviation of normal RR intervals (SDNN); (2) the square root of the mean of the squared differences between adjacent normal RR intervals (r-MSSD), and the number of pairs of adjacent NN intervals differing by more than 50 ms divided by the total number of all NN intervals (PNN50). Frequency domain outcomes will include HF and LF. The low frequency (LF)/high frequency (HF) ratio will also be considered as a frequency domain outcome reflecting the balance of the sympathetic vs. parasympathetic components of the nervous system.

4.6. Sample Size and Power Calculation: The study design is a randomized block with 12 repeated measurements per block (4 exposures x 3 times points each), the blocks being the subjects.

We present here a power calculation for the primary endpoints, brachial artery diameter (BAD) change and diastolic blood pressure change. All data will be paired, with each pollutant response being compared to each individual's filtered air response.

Our sample size calculation uses the traditional formula:

where the effect size $\Delta$ is the difference across exposure days of the baseline-post differences, and is the variance of the difference of these differences. For BAD, we have used data from a previously published study of 24 individuals[24]. BAD was measured before exposure (baseline) and immediately following exposure (post). For each subject, the baseline to post exposure absolute change in BAD was calculated. Following CAP + ozone exposure, there was a decrease of 0.092 ± 0.152 mm (mean ± SD) in BAD vs. an increase of 0.009 ± 0.181 mm following FA exposure. The corresponding mean we observed was 0.101 ± 0.215 mm (variance of 0.046). Using the of 0.046 and a conservative estimate of 0.09 mm for the size effect ($\Delta$), the following sample size result was derived. With 50 subjects we will have an 80% chance at the 5% significance level of detecting a $\Delta$ BAD of at least 0.1 mm between CAP and filtered air (FA) exposures.

3-11

In our blood pressure abstract[25], blood pressure measures included a 0-hr baseline and 2-hr measure used to calculate the 2-hr change in each exposure. The outcome $\Delta$ = (post-pre CAP) − (post-pre FA) will also be used. With the following criteria (as determined above), n=50, $\alpha$=0.05, 1-$\beta$=0.80, we will have the ability to detect a difference of $\Delta$=4.2 mm Hg between CAP and FA exposures (variance = 116.7 calculated from n=25 paired data from the published abstract[25]).

4.7. Statistical Analyses: Initial analysis of the data will include univariate explorations of all variables, using histograms, summary statistics, and outlier detection. Descriptive statistics will be calculated by exposure group and size fraction to enable visual inspection of differences among exposure groups. Scatterplots will be constructed to explore bivariate relationships among an outcome and continuous exposures. Data transformations will be considered as a means of satisfying regression assumptions of normality, linearity, and homoskedastic variances. As outlined in the Biostatistics Core, formal statistical modelling will follow a multi-tiered strategy of conducting analyses using exposure metrics of increasing sensitivity. The first stage will employ repeated measures ANOVA models containing a random effect for subject and a categorical variable for the four exposure groups (i.e., the difference size fractions), to assess differences among groups (gender will also be considered in the model). Standard multiple comparisons procedures, such as Dunnett's procedures, will be employed to adjust for making multiple comparisons among multiple exposure groups. Randomization as part of the study design (see Section 4.1) will allow us to separate exposure effects from time effects. Second, to assess relationships between an outcome and CAPs mass or individual components concentrations, single pollutant analyses will be conducted. A separate linear mixed regression model will be fit using biologic response as the dependent variable, subject as a random effect, and either mass, particle number, diameter or a single elemental concentration as the exposure metric in the model allowing for a separate slope for each size fraction. Third, to the extent possible given the correlation structure among PM constituents, multiple pollutant regression analyses will be conducted to investigate the joint effects of multiple components of PM. For outcomes recorded semi-continuously within an exposure, hierarchical linear models will be developed to account for the multiple levels (time within exposure within subject) of data. Such techniques allow assessment of differences among within-exposure slopes across exposure groups. Exposure levels of gaseous co-pollutants (carbon monoxide, $CO_2$, NO, $NO_2$, $SO_2$, NMCH, $O_3$), temperature and relative humidity, and day-before/day-after personal exposures levels ($PM_{2.5}$, BC, elements, $SO_4^{2-}$, $NO_2$) will be included in preliminary statistical analyses as potential effect modifiers or confounders. For a complete description of these methods, see Sections 3 and 4 of the Biostatistics Core.

4.8. Subjects: Subjects will be 18-50 year old non-smokers, 25 males and 25 females, without cardiovascular disease, hypertension (BP>140/90 mm Hg) or diabetes. All subjects will be free of lipid medication use or inhaled/oral corticosteroids and free of respiratory tract infections for at least three weeks prior to exposure testing. Participants will be recruited using advertisements placed around the University of Toronto campus and surrounding area as has we have done in previous studies[24,25]. Subjects will be offered remuneration for their time based on minimum hourly wages and amounts used in previous studies.
3-12

4.9. Safety Risks for Subjects and Ethical Considerations: All of the procedures and tests we will use are commonly done in respiratory/vascular research centres. Acute exposure to high concentrations of particles from ambient air has been reported to result in cough, shortness of breath, chest discomfort or headache. There is a possibility that subjects may

get a cold a few days following exposure. However, permanent health effects have not been reported from acute exposures to concentrations of particles that will be used in this study. We have already conducted exposures to fine CAPs at concentrations similar to those proposed in the current study, for over 100 subjects and have observed only minor complaints (such as headaches and mild respiratory tract irritant symptoms). Furthermore, we have found that these minor complaints were associated with the participants' perceptions of being exposed to a pollutant, as opposed to actual exposures[70].

To date, no subjects have asked to discontinue testing. Subjects will be monitored continuously during the exposure through windows, and by ECG and pulmonary function testing. A physician will be available at all times and will be equipped with suitable rescue medications to deal with possible adverse reactions. As well, both physician and subject will have the power to discontinue the exposure for that day, should any unexpected reactions occur. Finally, the protocol will be submitted to a Human Ethics Review Committee of the Office of Research Administration, Research Ethics Board of the University of Toronto and St. Michael's Hospital. Also it will be submitted to the HSPH Human Subject Committee for approval. We have had approvals for protocols with similar 2-hr $PM_{2.5}$ CAPs and $O_3$ human exposures.

## 5. EXPECTED RESULTS

The information produced from this study will be of considerable public health importance to PM risk management. This study will be the first of its kind to systematically examine the effects fine, ultrafine and coarse particles on cardiovascular hemodynamics and the autonomic nervous system. This will make it possible to provide insight into the mechanisms of PM-induced cardiovascular impairment and will be of significant value to the development of future hypothesis driven studies. Furthermore, the controlled human exposure design will complement the proposed Bus Study (Project 2) and the NAS Study (Project 1) in evaluating the relative impact of particles from motor vehicle emissions on cardiovascular outcomes. It will also complement our proposed animal study of vascular and inflammatory effects of particles (Project 4).

## 6. GENERAL PROJECT INFORMATION

6.1. Collaborations Among Participating Groups: The proposed study is a product of several previous collaborations between the University of Toronto Gage Occupational and Environmental Health Unit investigators (Silverman, JR Brook, Corey), the HSPH group (Koutrakis, Gold, Speizer and Godleski) and the University of Michigan (RD Brook). In 1998, Toronto researchers began conducting human exposure studies to $PM_{2.5}$ using the Harvard Particle Concentrator, which was designed and assembled in 1996 by Koutrakis et al. The new facility to be used for the proposed study will use three concentrator systems also designed and provided by Koutrakis et al. The collaboration between Toronto (Silverman and JR Brook) and Michigan (RD Brook et al.) has resulted in two research projects: funded by Canada's Toxic Substance Research Initiative and by US EPA STAR. Silverman and Gold have also collaborated

3-13

on conducting HRV measurements for human CAPs exposure studies, similar to those proposed in this project. Further analyses in collaboration are currently planned. Dr. Koutrakis has also collaborated in the past with others in the Toronto group (Broder, Sass-Kortsak, Purdham, JR Brook) in personal exposure monitoring studies using the Harvard personal monitors.

6.2. Study Personnel: The Principal Investigator, Dr. Frances Silverman, has extensive experience with controlled human exposure studies examining the pulmonary health effects, and more recently the cardiovascular effects, of air pollution. She will be the liaison with the other co-investigators. She will oversee all of the technical and administrative aspects of the project as well as overall data analyses, interpretation and report and manuscript preparation. Mr. Bruce Urch, study co-ordinator (with over 25 years experience with similar exposure studies) will be responsible for the day-to-day management of the study. Dr. Paul Corey, the study statistician, from the University of Toronto (Gage), will supervise the study randomization and the data management and will work closely with the statistical team at HSPH (Dr. Brent Coull). Dr. Diane Gold, Co-Principal Investigator from HSPH, has experience in the evaluation of air pollution effects on vascular outcomes (e.g., blood pressure, FMD) and on ECG outcomes. In the proposed study, Dr. Gold will function as the main liaison between the HSPH and University of Toronto groups. She will also participate in overall data analysis and interpretation, in collaboration with EPA Center Grant investigators, specifically Drs. Schwarz and Zannobetti. Dr. Jeffrey R. Brook, from Environment Canada, Toronto, will be responsible for the collection of particulate samples (collected at the Gage) for mass concentration, ionic constituents and total carbon. Dr. Robert D. Brook, from the University of Michigan will be responsible for analyzing and advising on all hemodynamic and vascular endothelial function measurements, as well as providing data analysis and interpretation. Dr. Julie Kovach, from the University of Michigan will be responsible for analyzing all echocardiography images/Doppler analyses and will provide expertise/consultation in cardiac hemodynamics. Dr. James Scott, from the University of Toronto, will be responsible for the development and implementation of techniques for the analysis of CAPs exposures for endotoxin and glucans.

6.3. Facility: The study will be performed utilizing the human air pollution exposure facility at the Gage Occupational and Environmental Health Unit of St. Michael's Hospital, University of Toronto. This facility presently has a fine particle concentrator that was developed in collaboration with HSPH[53,56,68,69]. Our research team has extensive experience in using this system to study numerous health outcomes, many of which have included minimally invasive and novel (to the air pollution field) techniques[24,25]. New funding from the Canadian Foundation for Innovation (CFI) is supporting the construction of an improved fine particle concentrator and new coarse and ultrafine particle concentrators[54,55]. These concentrators will be developed in collaboration with HSPH and will be operational by summer 2005.

6.4. Project Schedule: Year 1 will be dedicated towards the testing of new equipment, training of the echocardiography technologist and set-up/finalization of all protocols. In years 2-5 we will perform exposures. The exposure phase will begin with subject recruitment by advertisements placed throughout the Toronto area, screening of respondents, determination of eligibility (based on inclusion/exclusion criteria, physical

examination, blood test for lipids and glucose, pulmonary function, resting ECG) and obtaining consent for participation. Subjects will be recruited at the rate of ~12-13 per year for a total of ~ 50 exposures in each of the 4 years. 3-14

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3-19